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Bioconjugates to specifically render inhibitors water-soluble†

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Polymer–peptide conjugates are utilised to render small-molecule inhibitors of the kinase IspE, a promising new antimalarial target, water-soluble. It is shown that the peptide sequence of poly(ethylene oxide)–peptide conjugates can be tailored to mediate specific interactions with the ligands, allowing the solubilisation of even highly challenging inhibitors. This strategy avoids compromising the inhibitor structure to afford water solubility and potentially enables the exploration of a greater structural space of both inhibitors and drugs.

Structure-based inhibitor design constitutes an attractive, alternative approach to high-throughput screening. It has been successfully applied to the development of efficient and selective inhibitors of a number of drug targets such as BCR-Abl protein tyrosine kinase, leading to *gleevec* used in the treatment of chronic myeloid leukemia.¹ In addition to protein kinases, other kinases can also be interesting drug targets. An example is the kinase IspE,² an essential enzyme involved in the biosynthesis of the universal isoprenoid precursors in important pathogens³ and recognised target for the development of drugs against infectious diseases such as malaria and tuberculosis.⁴

The “Lipinski rule of five”⁵ empirically confines the space for drug-like compounds to low-molecular-weight entities with rather lipophilic character. Active sites of enzymes are frequently embedded in hydrophobic pockets of the protein, generating an environment where specific polar interactions are highly prominent. During drug development, an increase in affinity is often achieved with a concomitant increase in hydrophobicity. The resulting highly potent inhibitors often suffer from low water solubility, and can therefore not be subject to *in vitro* assays. To overcome this difficulty, several approaches have been explored to predict the water solubility of drug candidates.⁶ These techniques include the utilisation of numerical increments associated with known molecular fragments⁷ or *Monte Carlo* simulations.⁸ Even if a reliable prediction can be found to foresee the water solubility of potential inhibitors, a time-consuming optimisation of the molecular structure is required. Alternative strategies enhance the water solubility of drugs by

formulation approaches using, for instance, solubilising agents such as cyclodextrins,⁹ phospholipids¹⁰ or polymeric surfactants *e.g.* pluronics.¹¹

Recent advances in the field of polymer–peptide conjugates have paved the way to specific materials-science applications.¹² It was demonstrated that the peptide sequence in such bioconjugates can act as a monodisperse functional segment, displaying specific interactions programmed into the amino-acid sequence. This was exploited to (i) programme microstructure formation, (ii) control biomineralisation or silica morphogenesis, (iii) regulate the compactisation of plasmid DNA or (iv) transport cyostatica to metastases in the lymphatic system.¹³ If the interaction potential of peptide segments in polymer–peptide conjugates can be fine-tuned, the development of a generic specific solubilisation strategy, in which the peptide mediates carrier–drug interactions, can be envisaged.

Here, we present our initial investigation on the development of specific solubilisers to render potent inhibitors of IspE water-soluble.

The approach presented exhibits clear advantages compared to established strategies, where either poly(ethylene oxide) (PEO) is simply added to often rather complex drug formulations or functional PEO is covalently conjugated to the drug entity. While the former approach suffers from dilution problems, the latter implies the modification of the drug, generating a new chemical entity. This however, needs to be subject to a new validation process. The strategy proposed herein does not encounter similar problems as the drug is not covalently modified. An additional benefit is the fact that only the peptide sequence might have to be altered. This involves straightforward automated peptide synthesis, which can be considered as much less expensive and faster than a classical, synthetic optimisation cycle of the inhibitor.

The structure-based design of IspE inhibitors was aided by the modelling programme MOLOC (*cf.* ESI†).¹⁵ A new class of bisubstrate inhibitors was developed (Fig. 1, inhibitors **I–II**), targeting both the substrate- and the ATP-binding pockets of the enzyme IspE with the potential for higher selectivity and higher binding affinity (*cf.* Fig. S1, ESI†).¹⁶ In addition, some previously designed small-molecule inhibitors, which only occupy the substrate-binding pocket of IspE kinase, were also included in this investigation (*cf.* Fig. 1, inhibitors **III–IV**).¹⁷

Bisubstrate inhibitors **I** and **II** were entirely insoluble under the enzymatic assay conditions, whereas the monosubstrate inhibitors **III** and **IV** were poorly soluble, yet their IC₅₀ values¹⁸ could be determined.¹⁷ In these *in vitro* assays, the assistance of organic cosolvents such as up to 10 vol% DMSO was required to afford sufficient ligand solubility. This cosolvent-addition strategy proved to be unsuccessful for the class of bisubstrate inhibitors **I** and **II**. Presumably, the general solubility issue can be ascribed to the cytosine moiety featured by all derivatives.^{17b} The remarkably low water solubility of the ligands **I** and **II** in particular can be ascribed to the rigid structure and

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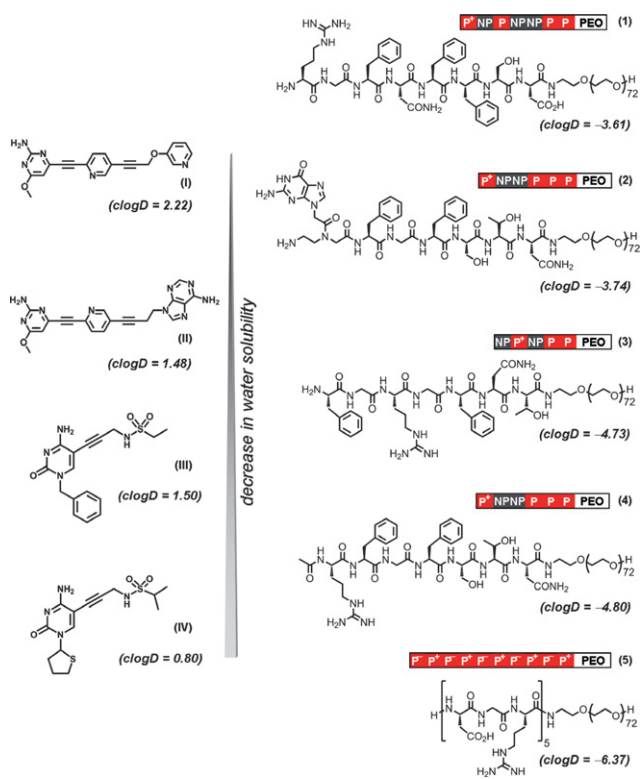


Fig. 1 IspE inhibitors (left) and a library of polymer-peptide conjugates screened to specifically solubilise the inhibitors (right) ordered by water solubility as predicted by clogD values.¹⁴

pronounced hydrophobicity, as indicated by the high partition coefficients (*cf.* clogD values provided in Fig. 1).

To overcome the solubility issues without an additional design cycle and the coupled synthetic effort, *de novo* designed polymer-peptide conjugates have been investigated to render inhibitors water-soluble.

A total of five tailor-made peptide sequences were synthesised and conjugated to PEO *via* an inverse conjugation approach (*cf.* Fig. 1, polymers **1–5** and ESI[†]).¹⁹ The peptide sequences cover a broad spectrum of polarity (from **1** which is rather nonpolar to **5** which is highly polar) and differ in the hydrophobicity–hydrophilicity pattern. As general design criteria, amino-acid residues were selected, providing hydrophobic contacts [phenylalanine (Phe)], *Coulombic* interactions [arginine (Arg) or aspartic acid (Asp)] and nonionic hydrogen bonding [asparagine (Asn), serine (Ser) and threonine (Thr)]. Sufficient conformational flexibility was ensured by glycine (Gly) as spacer residue. Conceptionally, this allows to position selective interaction capabilities along the peptide strand, assembling a segment that modulates the interactions with the inhibitor entity. The PEO block was selected to provide both water solubility and shielding of the polymer–inhibitor complexes.

The solubilisation experiments were performed by dissolving one equivalent of inhibitor and one to four equivalents of the polymeric carrier in a small amount of DMSO. The resulting homogeneous solution was diluted to 3.8 vol% DMSO by the addition of water with a rate of 80 $\mu\text{L/h}$. A first screen of the monosubstrate inhibitors **III** and **IV** with different carrier conjugates (**1–5**) indicated a straightforward solubilisation of the inhibitors by the most polar carrier **5** (*cf.* Table 1). This was to be expected due to the compact, polar

nature of the inhibitors, in which the sulfonamide moiety participates in extensive hydrogen-bonding interactions. It is noteworthy that the overall content of DMSO could be reduced significantly from 10 to 1.3 vol% in the enzyme assay, if compared to the cosolvent strategy, in which the polymeric carrier is absent. In all cases, the most polar carrier **5**, exhibiting a zwitterionic peptide, results in very successful solubilisation (Table 1). However, inhibitor **IV** is also solubilised by the rather apolar carriers **1** and **3**. This reflects rather low specificity for the class of monosubstrate inhibitors.

With this promising result in hand, the design of tailor-made peptide sequences to afford carriers that would solubilise even the most challenging bisubstrate inhibitors **I** and **II** was addressed (Fig. 2a). To generate a sufficient interface between the carrier and the inhibitors, peptide strands were composed in a straightforward manner. Arg residues should serve to bind to the substituted pyrimidine ring, which acts as cytosine analogue. The benzylic side chain of the aromatic amino acid Phe should establish π – π stacking interactions with the alkyne linkers, and polar amino-acid residues should satisfy the heteroatoms of the pyridine rings and the ether linker. The peptide sequence of carrier **2** was not terminated with an Arg residue as present in carrier **4** but with a peptide–nucleic-acid (PNA) monomer hypothesised to form a Watson–Crick base pair with the cytosine moiety. By analogy, the guanine–PNA monomer might form hydrogen-bonding contacts to the cytosine analogue.

Besides considering these straightforward design principles, the modelling software MOLOC¹⁵ was used to assist peptide design (Fig. 2b). To model potential sequences, the equilibrated inhibitor was set stationary while letting the peptide freely optimise around the inhibitor. Considering hydrogen-bond acceptor and donor sites as well as hydrophobic and hydrophilic regions, it was possible to generate a peptide sequence (**1**) that should be complementary to inhibitor **I**.

Solubilisation of the bisubstrate inhibitors by the set of PEO–peptide conjugates was studied by adopting similar DMSO–water dilution protocols as described for the monosubstrate inhibitors. The results are summarised in Table 1. As expected, the solubilisation of ligands **I** and **II** proved to be more challenging compared to the monosubstrate inhibitors. The results are clearly sensitive to fine differences in functionality and polarity patterns in the peptide sequences. This suggests a higher selectivity of the interactions in these polymer–inhibitor complexes. While inhibitor **II** could only be solubilised as a solution exhibiting slight opaqueness, for **I** only one positive hit was found. Despite the structural analogies between ligands **I** and **II**, the former was solubilised by highly polar carriers (**4** and **5**), whereas effective solubilisers for the latter could be found at the other end of the polarity spectrum (**1** and **3**). Interestingly, carrier **1** that was based on simple computer-assisted design could indeed effectively enhance the solubility of the bisubstrate inhibitor **I**. However, careful studies are required to solidify the strategy. It should be highlighted that the current picture of the inhibitor–carrier complex is certainly idealised as suggested by the fact that stoichiometry is important. While inhibitor **I** is solubilised as a slightly opaque solution if one equivalent of **1** is provided, addition of four equivalents of the carrier polymer leads to a clear solution.

The solutions of solubilised inhibitors were characterised by means of UV-vis spectroscopy and dynamic light scattering. UV-vis spectroscopy confirmed the ease of solubilisation of the monosubstrate inhibitors (**III** and **IV**). Assuming an extinction coefficient of the inhibitors that is independent of the solvent (methanol and water), the

Table 1 Solubilisation experiments and *in vitro* evaluation^a

Inhibitor	Polymer carrier					IC ₅₀ [μM]	
	1	2	3	4	5	Without carrier ^c	With carrier ^f
I	++ ^{bf}	— ^b	+	—	—	n.d.	8.7
II	—	—	—	+ ^b	+ ^b	n.d.	n.d.
III	—	—	—	—	++ ^{bf}	79.0 ^d	n.d. ^e
IV	++	—	++ ^{bf}	—	++	8.0	8.0

^a Solubilisation: —: solid precipitate; —: slightly turbid; +: slightly opaque; ++: clear solution (conditions: formulation *via* dilution approach (100% DMSO → 3.8 vol% DMSO in water; 16 h, 25 °C). ^b Concentrations of solubilised inhibitors in mg/mL: $c[\text{I/I}]_{\text{app.}} = 0.36$; $c[\text{II/4}]_{\text{app.}} = 0.20$; $c[\text{II/5}]_{\text{app.}} = 0.18$; $c[\text{III/5}]_{\text{app.}} = 0.08$; $c[\text{IV/3}]_{\text{app.}} = 0.39$; n.d. not determined due to insolubility. ^c Cosolvent-assisted solubilisation. ^d For comparison: 30% inhibition at 34 μM. ^e 30% inhibition at 47 μM. ^f Samples used for the inhibition study.

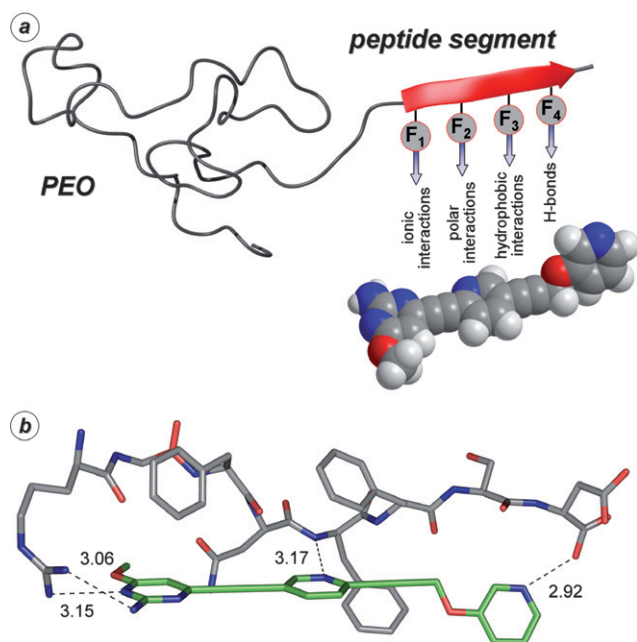


Fig. 2 (a) Illustration of the concept applying PEO-peptide conjugates as specific solubilisers to render inhibitors of IspE water-soluble. While the peptide segment modulates interactions with the inhibitor, the PEO-block provides water solubility (F_{1–4} indicate side-chain functionalities of amino acids). (b) Low-level molecular simulation of the peptide-inhibitor complex [I/I], showing a local minimum structure. Interactions are mediated by Arg1 and Asn4 providing hydrogen-bonding interactions, Phe6 giving hydrophobic contacts and Asp8 leading to ionic interactions (the PEO block was not considered).

apparent inhibitor concentration could be estimated. The quantitative UV measurements provided $c[\text{III}]_{\text{app.}} = 0.08$ mg/mL and $c[\text{IV}]_{\text{app.}} = 0.39$ mg/mL, which meet the expected concentration range ($c[\text{III/5}]_{\text{th.}} = 0.1$ mg/mL and $c[\text{IV/3}]_{\text{th.}} = 0.5$ mg/mL, respectively). The estimated values of the bisubstrate inhibitors have been slightly lower with $c[\text{I}]_{\text{app.}} = 0.36$ mg/mL and $c[\text{II}]_{\text{app.}} = 0.18$ – 0.20 mg/mL ($c[\text{I/I}]_{\text{th.}} = 0.5$ mg/mL and $c[\text{II/4}]_{\text{th.}} = c[\text{II/5}]_{\text{th.}} = 0.25$ mg/mL, respectively). Light scattering with a *Nicomp Particle Sizer* shows structures beyond the detection limit, suggesting that the dominating structures are well below 10 nm. A more detailed investigation of the kinetically controlled aggregates and in particular of the fine structures of the peptide-inhibitor contacts have to be performed by advanced scattering and NMR experiments.

These results demonstrate that polymer-peptide conjugates can be successfully used to improve the water solubility of drug-like compounds. For this approach to be viable, it had to be demonstrated that the polymer does not affect the biological activity of the inhibitors. A series of initial control experiments demonstrated that the carriers (1–5) do not display inhibitory activity.

The IC₅₀ values of the carrier-inhibitor complexes were determined, employing the established photometric assay as indicated in Table 1 (footnote f).²⁰ The IC₅₀ values of the solubilised monosubstrate inhibitors **III** and **IV** are in the micromolar range and agree well within the error of the assay with values obtained previously for the isolated inhibitors.¹⁷ The latter values were determined in the presence of 10 vol% of DMSO as an organic cosolvent to solubilise the inhibitors. Use of PEO-peptide conjugates allowed to decrease the amount of DMSO down to 1.3% in the enzyme assay.

The fact that the IC₅₀ value of bisubstrate inhibitor **I** could be determined for the first time, can be considered an achievement. The inhibitory activity of 8.7 μM lies in the range of the most potent monosubstrate inhibitors described to date. Given that the inhibitor is far from being an optimised system, this initial result is very promising and sets the stage for further design cycles. In the meantime, further optimisation of the carrier and the formulation protocols are currently ongoing to address other promising mono- and bisubstrate inhibitors with low or no water solubility, also in view of extending the solubilisation strategy to enzyme-inhibitor co-crystallisation studies.

In summary, the application of polymer-peptide conjugates as selective solubilisers to render small-molecule inhibitors water-soluble has been reported. The approach presented enables the solubilisation of mono- and novel bisubstrate inhibitors for IspE, an essential kinase in the non-mevalonate pathway of isoprenoid biosynthesis and a recognised drug target in the fight against infectious diseases such as malaria or tuberculosis. A set of PEO-peptide conjugates were screened to solubilise four different kinase inhibitors that show low to no water solubility. While the peptide segment in the conjugate could be programmed to modulate specific interactions with the inhibitors, the PEO segment provides the complexes with water solubility. The polymer-peptide conjugates afford water-soluble inhibitors without affecting the biological activity of the inhibitors analysed in the context of this study. Besides enhanced practicality, the new approach will enable the use of a larger variety of structures, given that the design of potential inhibitors no longer has to be guided by features known to afford water solubility but rather by promising recognition motifs. This will certainly expand the window of drug

candidates toward highly hydrophobic compounds. Moreover, advanced functions can be integrated into the carrier structure, setting the stage for controlled release of the cargo or strategies that allow the homing of the carrier to address certain tissues or cells in a specific manner.

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